Cdc6-mediated regulation of the initiation of eukaryotie DNA replication

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学位論文題目

Cdc6-mediated Regulation of Eukaryotic DNA Replication（Cdc6 による真核生物 DNA 複製の制御）

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論 文 内 容 要 旨

Given the immense size and complexity of eukaryotic genomes, duplication of chromosomal DNA presents a significant challenge for the cell. Therefore, the initiation of DNA replication must be tightly regulated to ensure stable maintenance of the genome. This is achieved at first by the strict sequential binding of ORC, Cdc6, Cdt1 and Mcm2-7 forming the pre-replicative complex (pre-RC) and thereby licensing the origin. As replication initiates, Mcm2-7 complexes are released from the origins and move along with or ahead of the replication forks, possibly functioning as DNA helicases. The majority of work in recent years has focused on identifying the relevant players involved in forming and activating pre-RCs, determining the order in which they associate with origins, and characterizing their cell cycle regulation. However, our understanding of the mechanism of pre-RC assembly, and how pre-RC prepares the origin DNA for initiation remain limited. Cdc6 is one of the proteins functioning for the licensing, and is readily destabilized from chromatin after Mcm2-7 loading and rebinds to chromatin later in S phase. This dynamic loading-dissociation-reloading behavior of Cdc6 creates a window of time when Cdc6 is almost absent or loosely bound to chromatin. In this study, I focused on Cdc6 and analyzed the consequences of deregulating Cdc6 after origin licensing at molecular level in order to elucidate the significance of its regulation during the course of DNA replication using *Xenopus* egg extract cell-free system.

In order to directly investigate the consequences of deregulation of Cdc6, I purified recombinant wild-type GST-Cdc6 from *Escherichia coli* and added it to *Xenopus* egg extracts to create a state in which more Cdc6 protein binds on chromatin for longer period even after origins have been licensed. Given the fact that Cdc6 is a positive regulator of the cell cycle, it has been presumed that an excess of Cdc6 would result in overreplication or rereplication. Surprisingly, this study shows that presence of excess Cdc6 inhibited DNA replication independent of caffeine-sensitive checkpoint pathways in *Xenopus* egg extracts, when its concentration was a few times higher than the endogenous level. The inhibition by the excess Cdc6 occurred after chromatin binding of Cdc7 and before Cdk2-dependent steps as the addition of Cdc6 hardly influenced functional pre-RC formation, the chromatin binding of Cdk2, or DNA synthesis after release from inhibition of Cdk2 activity or elongation of nascent DNA. The treatment, however, suppressed phosphorylation of Cdc7 and Cdc7-dependent hyperphosphorylation of Mcm2-7 subunits on chromatin, especially hyperphosphorylation of Mcm4 (Figure 1). Remarkably, Cdc6 itself does not directly inhibit phosphorylation of recombinant Mcm2-4-6-7 complex by Cdc7 in purified systems, rather modulates phosporylation status of Mcm2-7 on chromatin context. Taken together, this work suggests that Cdc6 on chromatin acts as a modulator of Cdc7-mediated phosphorylation of Mcm2-7 and thus destabilization of Cdc6 from chromatin after licensing is a key event in the cell cycle ensuring proper transition to the initiation of DNA replication.

In order to gain further insights into Cdc6-mediated regulation, I next sought to analyze the structural basis for the inhibition of DNA replication by Cdc6. A growing body of evidence indicates that Cdc6 is phosphorylated by cyclin-dependent kinases (CDKs) and acetylated by a histone acetyltransferase (HAT), Gcn5, by which progression

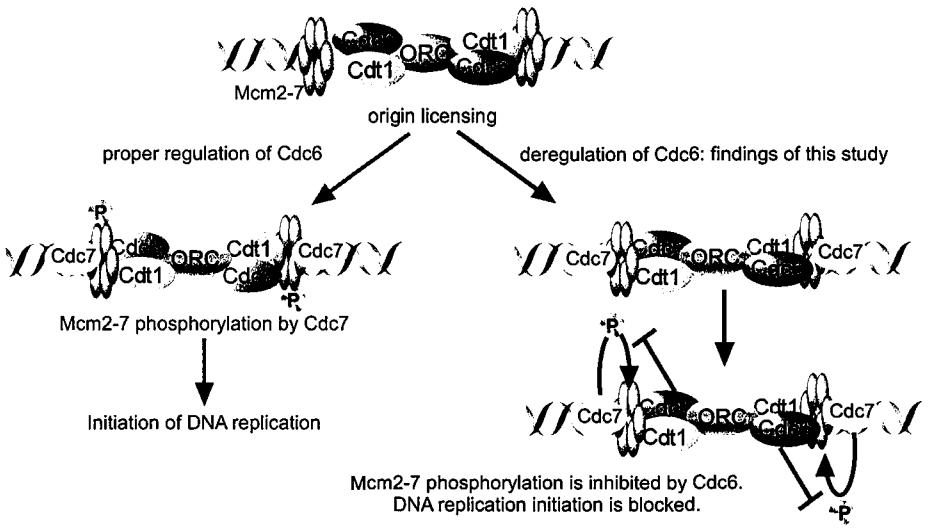

Figure 1. Regulation of DNA replication initialation by Cdc6.

of the cell cycle is likely to be regulated. Given that the post-transcriptional modifications of Cdc6 play crucial roles in cell cycle progression, mutations of the modification sites in Cdc6 might affect suppression of DNA replication initiation caused by the addition of recombinant Cdc6. Thus, I constructed a panel of truncated and mutated proteins of Cdc6 and investigated their effects on DNA replication initiation. The results suggested that the domain necessary to suppress DNA replication initiation lies in the N-terminal 1-186 amino acids of Cdc6. Further analyses suggested that acetylation of Cdc6 on its N-terminus is crucial for proper transition to the initiation of DNA replication, and that acetylation of Cdc6 prior to origin licensing is detrimental to DNA replication. These results reveal a fine tuning of Cdc6 activity before and after origin licensing thereby ensuring a strict regulation of DNA replication initiation.

Finally, I sought to analyze the cross-talk between Cdc6 and other proteins interacting with Mcm4 during DNA replication and came up with a serendipitous discovery regarding the chromatin binding dynamics of one of histone chaperones, facilitates-chromatin-transcription (FACT) complex, during DNA replication. In eukaryotes, FACT acts as a histone chaperone that affects nuclear DNA transactions in a chromatin context. Despite that the involvement of FACT in DNA replication has been reported, a clear understanding of its behavior and biochemical function during DNA replication still remains elusive. In this study, I find that FACT has at least two distinct chromatin binding phases; 1) a rapid chromatin binding independent of DNA replication, and 2) a second mode of chromatin binding initiating after origin licensing. Intriguingly, early-binding FACT dissociated from chromatin when DNA replication was blocked at licensed state before origin firing by the addition of Cdc6. Cdc6-induced removal of FACT was blocked by co-addition of geminin, a licensing inhibitor, but not by suppressing the activity of DNA polymerases, CDK or Cdc7. Furthermore, chromatin transfer experiments revealed that impairing the latter binding of FACT severely compromises DNA replication activity. These results suggest that even though FACT has a rapid chromatin binding activity, the binding pattern of FACT on chromatin undergoes a change coupled to origin

licensing, which may contribute to the establishment of its functional link to DNA replication machinery.

The main achievement of this research is the discovery of a novel regulatory mechanism of the initiation of DNA replication signifying the importance of a strict regulation of Cdc6 function during the course of DNA replication. Furthermore, by using Cdc6 as a novel tool to halt DNA replication before initiation, this study uncovers the chromatin binding dynamics of FACT complex which has been long-proposed to have essential function during DNA replication. The next challenge in the future will be to decipher how this regulation of DNA replication initiation by Cdc6 is overcome during the progression of carcinogenesis where in many cases Cdc6 is overexpressed.

References

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審 査 結 果 の 要 旨

遺伝情報の複製と分配は生命の根本事象であり，DNA 複製機構の解明は分子生物学における最重要課題の１つである。論文提出者 Lena Rani Kundu 氏は，真核生物における DNA 複製の分子機構を解明する目的で，複製因子 Cdc6 の機能解析を行った。アフリカツメガエルの卵抽出液を用いた試験管内 DNA 複製反応に精製 Cdc6 を過剰に加えた結果，複製反応が阻害されることを見いだした。次に，複製開始複合体形成（Origin licensing）から複製伸長反応の開始（Origin firing）にいたる DNA 複製反応のどの段階が，精製 Cdc6 の添加により阻害されるかを解析した。その結果，Origin licensing 反応以降かつ CDK による Origin firing 以前の反応が，精製 Cdc6 の添加により阻害されることを明らかにした。さらに Origin firing に必須な Mcm2-7 複合体のリン酸化について解析を行った結果，クロマチン上の Cdc7 による Mcm4 のリン酸化が阻害された。以上の結果により，DNA 複製の初期反応において，Origin licensing 後に Cdc6 が速やかに開始複合体から解離する事が，Cdc7 による Mcm2-7 複合体のリン酸化と，その後の Origin firing に極めて重要であることが示された。論文提出者は以上の内容を原著論文（Kundu, *et. al.*, ***Nucleic Acids Research***, 2010, 38: 5409-18.）として発表している。

次に試験管内複製反応の阻害活性に重要な，Cdc6 のドメインの解析を行い，アミノ末端ドメインが，阻害活性に重要であることを明らかにした。このアミノ末端ドメインは HAT（ヒストンアセチル化酵素）によりアセチル化修飾を受けることがヒト HeLa 細胞において報告されていたため，HAT 阻害剤である VPA の効果を検討した。その結果 VPA 存在下では，精製 Cdc6 の添加によって試験管内複製反応が阻害されなくなった。これは，Origin licensing 後の複製開始複合体からの Cdc6 の速やかな解離に，Cdc6 のアセチル化が重要であることを示唆する結果である。最後に論文提出者は，DNA 複製反応に重要であり，Mcm 複合体のヘリケース活性に必須であるヒストンシャペロン FACT について，そのクロマチン結合の制御機構を解析した。その結果，複製伸長反応の開始（Origin firing）後に FACT がクロマチンへ結合することが DNA 複製に重要である可能性を明らかにし，原著論文（Kundu, *et. al.*, ***Biochim Biophys Acta***, in press）を発表した。

以上の成果は，DNA 複製反応における Cdc6 を介した制御機構の重要性を明らかにするとともに，DNA 複製機構の全体像を理解する上でも重要な視点を提供するものである。

よって，本論文は博士（薬学）の学位論文として合格と認める。